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A comparison of protocols for passive and discriminative avoidance learning tasks in the domestic chick

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difference between passive avoidance and discriminative avoidance protocols and this may explain the differences in duration of the memory and the different stages. Is the OU task a more salient experience because of the novelty of the bead and therefore a 'stronger' learning experience? The different protocols may allow different questions to be addressed.

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*** Conflict of Interest**

There is no conflict of interest relevant to this manuscript.

Dr Stephen Dunnett
Brain Research Bulletin

28 November, 2007

Please find the final manuscript to be submitted for the Special Issue of Brain Research Bulletin on Brain mechanisms....

Yours truly

Marie Gibbs

Abstract

A one-trial learning task where chicks learn that a bead of a particular shape and/or colour has a bitter taste (100% Methyl anthranilate – MeA) and subsequently avoids it on test has been widely used by research groups across the world. However, there are some differences in the results reported by different research laboratories. One important difference is found when chicks are trained on a diluted bitter taste (10 or 20% MeA) such that memory is not consolidated and fades, e.g. memory lasts for 30 min at Monash and La Trobe Universities versus 4-6 hours at the Open University (OU). Differences in protocol that may explain this apparent discrepancy are whether the chicks have seen the bead before (novelty), and whether the colour or the shape of the bead is a more important feature. In this review, we discuss these and other factors that may contribute to the differences in the characteristics of memory processing between Monash and the OU, e.g. strain, hatchery or laboratory incubated chicks, age at training. It is clear that there is a difference between passive avoidance and discriminative avoidance protocols and this may explain the differences in duration of the memory and the different stages. Is the OU task a more salient experience because of the novelty of the bead and therefore a ‘stronger’ learning experience? The different protocols may allow different questions to be addressed.

1.Introduction

The single trial bead learning used by groups led by Gibbs and Ng at Monash University and Simon Crowe at La Trobe University (Australia) and Steven Rose and Radmila Mileusnic at the Open University (UK), Richard Andrew at the University of Sussex (UK) and others elsewhere in the world, has been purported to be a very simple highly reproducible task. It is, but only within each group and somewhat different results between the laboratories has made for difficulties of interpretation. In this paper we examine some of the factors that contribute to this variability. What seem to be subtle task differences to us, may be very important to the chick. We also canvas other factors that might be involved such as strain, source of chicks (commercial hatch vs laboratory incubators), differences in training and testing protocols, scoring and time schedules.

2.History

The one-trial passive avoidance learning task was first used in domestic chicks (*Gallus gallus domesticus*) by Art Cherkin in 1969. The advantage of using neonate chicks is that “they peck a suitable target but learn in one trial to avoid that target if it is coated with an aversive liquid when first pecked” [6]. The description of the learning task was published in the Proceedings of the National Academy of Sciences in a paper entitled ‘Kinetics of memory consolidation: role of amnesic treatment parameters’. Essentially, a chick was presented with a ‘microminiature lamp’ coated with liquid methyl anthranilate (MeA). The chicks were allowed 10 seconds to peck at this bead and when presented again with a dry target the chicks now avoided the bead. The learned avoidance persisted at least nine days. However, the number of chicks avoiding the lamp in the control groups varied from day to day and so an ‘induced’ peck score was used to correct for the day-to-day variation in control score. It is important to emphasize that the chicks would peck a dissimilar target proving that peck performance was unimpaired and the learned avoidance was not a generalized avoidance response.

Prior to the publication of this work of Cherkin’s in 1969, Marie Gibbs (then Watts) and Richard Mark at Monash University developed a modified version of this task. In 1971 MG was able to spend 6 months working in Cherkin’s laboratory in Sepulveda,

California and it was decided that the major differences between the two tasks were keeping chicks in isolation (Cherkin) and prior exposure to objects to peck at in pretraining (Monash). At Monash the chicks were kept in pairs to avoid the stress of isolation. The birds were extremely vocal when isolated, a problem Art Cherkin countered by using masking white noise at 76 dB. The other difference was that at Monash 'pretraining' was used with a clean version of the target to that was used in training, both to encourage chicks to peck at beads on the presentation into their cage, to reduce the possibility of frightening the chicks. Part of Cherkin's logic for keeping chicks isolated was that with two chicks together, there was the possibility that they could 'copy' each other. (This question has been dealt with by both Ng, Crowe and Gibbs [33] and by Johnston, Burne and Rose [28] albeit with differing conclusions). Using the different protocols, differences were found in the timing of when memory was vulnerable to interference by drugs and the timing of when memory faded in amnesia, ie differences in the duration of the memory stages found at Monash [31, 51]. These differences between the two research protocols at Sepulveda and Monash were never reconciled and the isolated, non-pretrained chick protocol was adopted by Rosenzweig, Bennett and others at the University of California at Berkley [11].

Around 1978 two other groups joined the 'passive' avoidance scene. Steven Rose at the OU, UK and Richard Andrew at Sussex University, UK. Both were introduced to the task by Gibbs and both initially used similar protocols i.e. keeping chicks in pairs and pretraining chicks to peck at beads prior to the training trial, but over the years each group has made changes to the original Gibbs and Ng protocol.

Since the early 1970's this one-trial 'passive avoidance' task for chicks has been used by a number of laboratories all over the world to explore many memory parameters, from biochemical and molecular correlates of memory, to screen memory enhancing agents and to study memory related phenomena such as memory lateralization, memory consolidation and retrieval, and memory reconsolidation. Features of the task which are considered to provide an advantage when looking for stages in memory and the underlying physiological and biochemical mechanisms have been described a number of

times eg [23, 41, 43].

The main features are :

- 1) The young chick is precocial and at hatch can see, hear and taste, its pecking and locomotor responses are well coordinated. Most importantly it can be trained on a number of different tasks that may be germane to its survival and it remembers them well.
- 2) The brain is relatively large for a small animal, weighing around 1 gm at hatch, comparable in size to a rat brain. This is an advantage for localization of intracerebral injections, for biochemical studies and for electrophysiological recordings.
- 3) The brain is covered by a soft unossified skull; this permits rapid injections without the necessity for anaesthesia (given close to learning anaesthesia can produce amnesia). This speed allows for precise and accurate timing of training and testing, with large n values, unlike in rodents.
- 4) Chicks do not respond to brain needle stick injury with electrical seizures, a limitation of attempting to make injections directly into brain tissue in rodents.
- 5) The virtual lack of any blood-brain barrier in these young birds allows for rapid entry of peripherally injected drugs into the brain. However, it must be remembered that these will then impact on all areas of the brain as well as in the periphery.
- 6) It is feasible to run up to 10 experimental conditions on one experimental day so that comparisons of treatments can be made using a single batch of chicks (160-240).
- 7) It is possible to tightly control the development of the chick during incubation, so that auditory and visual (light) stimuli can be controlled, monitored and manipulated [5, 38-40, 49, 50].
- 8) The sequence of the chicken genome has been published [26]. There are some significant overlaps between chick and human/mouse genomes, and in some instances oligos/antisense designed against one are effective against another and may indicate similar functions. However, there are many differences, as there are between mouse and human, so it has to be ascertained that the oligos and antisense markers are the same.
- 9) Finally, on the basis of molecular genetic studies [34] the nomenclature of the chick brain has been revised, revealing major organizational similarities between avian and mammalian brains. The names of brain regions now reflect the embryonic source of the

different brain regions and the comparability between mammalian and avian brain structures has been acknowledged [35, 36]. The mesopallium in birds possesses significant cognitive abilities and has an impressive behavioural repertoire of language development and cognitive functions.

As mentioned above, as seems inevitable when new experimenters take up and use a relatively simple task, differences were introduced into the Monash protocol and differences became apparent in the results. We now discuss some possible reasons for these.

Strain of chicks.

The strain of chick being used differs across countries. The OU use laboratory hatched ‘broiler chicks’ whereas Monash use ‘egg-laying strains’. The latter are quite often hybrids of the common egg-laying strains, however no strain is likely to be ‘pure’. In the commercial hatchery situation where the chicks bred for the purpose of laying eggs, the male birds are ‘redundant’ and can be purchased quite cheaply from the hatcheries. It is possible that there may be subtle differences in the behaviour and the memory processes of chicks from meat laying strains and those from egg laying strains, and these could potentially produce minor alterations in behavioural responses, but it is clear that the major findings are the same at both Monash and the OU. Burne and Rose [4] compared many aspects of the protocols used at Monash and the OU using chicks from a meat strain, and found that they could replicate the essential findings of both protocols in terms of whether the chicks remembered or not after weakly-reinforced training. Thus it seems fairly unlikely that the differences in duration of recall can be ascribed to strain differences between chicks.

Very few significant sex differences have been recorded for this task, although care should be taken when hormones, particularly sex hormones, are being used to manipulate memory processing. This is one of the advantages of buying in male chicks from the poultry farm where they use experienced people to determine the sex of the chicks. Although the sex of laboratory incubated chicks can be ascertained (see Table 1) it is a rather time consuming operation to dissect the chicks and observe the gonads. In one

experiment (unpublished) female chicks were trained at Monash and found to have very similar memory vulnerability to drugs (ouabain and cycloheximide).

Hatchery vs laboratory incubated chicks.

Another possible factor in the protocols is whether the chicks were incubated and hatched at the University under known conditions or brought in from local hatcheries. Many prehatching factors are known to alter subsequent behaviour and even brain neurochemistry in chicks and thus may well influence responses in this task [27]. The convenience of buying in chicks from the hatchery where the conditions are uniform and the chicks hatch in large numbers has to be weighed against hatching them in the lab. The conditions in the hatchery of choice certainly need to be ascertained and factors like whether the eggs are exposed to light or not need to be determined. As we know, lateralization is dependent on light exposure around day 17 [52] and many hatcheries transfer their embryos from the incubator to the hatchery at this time. Memory in the hatchery chicks used at Monash show clear lateralization [20] like laboratory incubated chicks at both Monash and the OU.

Prior experience or pre-training protocols.

At the present time, the two/three most active laboratories using the single trial task (OU, UK and Monash and La Trobe Universities, Australia) report somewhat different results in terms of duration of memory. One of the major reasons for the differences in data in the different laboratories, probably lies in the differences in pre-training protocols [4], which impacts on the nature of the learning task itself and the consequent duration of memory, particularly in a weakly-reinforced (diluted MeA) version of the task.

Strength of methyl anthranilate and weakly-reinforced training.

Although Burne and Rose [4] consider that “there is, in general, agreement concerning the anatomical, biochemical, and cellular consequences of training on the strongly-reinforced task between the groups”, it is apparent that there are differences in the time course of memory formation, and these differences are most noticeable in the weakly-reinforced task [14, 19].

Weakly-reinforced memory: 0.25% anthranilate in water. The weakly-reinforced task was introduced in 1971 by Art Cherkin [7] where the methylantranilate (MA) on the bead was not 100% but was diluted to 1:400 (0.25%) in water. The extremely weak concentration of MA was necessitated by the insolubility of methylantranilate in water. Cherkin had reported that memory retention and avoidance of targets coated in this weak aversant was still present 6 hours after training, but was gone by 24 hr, a very similar result to that found at the OU). However, when this protocol was used for the first time with coloured beads and the Monash protocol, memory was lost after 40 min [19, 25]. At least part of the explanation for the differences in the duration of labile memory was that in Art Cherkin's experiments, the chicks were kept in isolation. Isolation of chicks is stressful and memory processing under stressful conditions leads to extension of memory stages or even to prevention of memory loss [17, 22, 29, 46, 47]. Indeed, in experiments in Art Cherkin's laboratory (Gibbs and Cherkin, unpublished) memory loss, using his protocol with strongly reinforced training, normally produced by giving the protein synthesis inhibitor cycloheximide 10 min after training, did not occur unless a second cycloheximide injection was given some 60 min after training, and memory loss after injection of ouabain did not occur until after 90 min. This is in stark contrast to training with paired chicks [23, 51] where memory loss occurred after 10 min following ouabain injection and after 30 min following a single injection of cycloheximide immediately after training. Nevertheless, it has to be pointed out that the results found by Cherkin on the duration of memory following weakly-reinforced training were very similar to those found by the OU researchers, ie memory lasted for 6-8 hours before fading. This suggests that isolation of chicks was not the sole factor responsible for the discrepancy between laboratories.

Weakly-reinforced memory: 10 and 20% methyl anthranilate in alcohol.

In 1989 Simon Crowe, extended on the experiments using weakly-reinforced training [12-15] and looked at the effect of different dilutions of MeA in alcohol. Memory retention following training on 0, 10, 20, 33 and 50% dilutions in alcohol varied according to the concentration of MeA. Control experiments showed that alcohol did not

produce avoidance (measured as discrimination ratio) at 5, 30 or 180 min after training, whereas training with 50% MeA resulted in high discrimination ratios at all three times. Although there was an absence of memory retention with both 10 or 20% anthranilate when memory was measured at 180 min, there was good memory with 20% anthranilate at 30 min but not with 10% anthranilate [14]. These results implied that there is a dose dependent effect of training ‘strength’ on memory retention, it is reflected in the duration of memory in labile storage rather than in the level of discrimination at 180 min. Increasing the number of training trials by presenting a bead dipped in 0.25% MeA (in water in experiments before the change to diluting it in alcohol) every 15 min had the effect of progressively extending the duration of memory, until eventually memory was consolidated [25]. Each time a new training trial occurred the memory appeared to ‘start again’ and progress going through short-term to intermediate memory, but each time the duration of the intermediate memory became longer.

Stages of memory

The essence of the three stages of memory model proposed by Mark and Watts in 1971 and then extended by Gibbs and Ng, was that there 3 stages of memory – short-term, intermediate and long-term memory. Following this work at La Trobe (Gibbs), behavioural experiments without drug intervention, showed that these 3 stages were delineated by transient retention losses of memory at 15 and 55 min after training. Although these dips have been looked for extensively at the OU they have not been seen. However, it is possible if they are very sharp dips, it would be possible to miss them. These dips are known to ‘shift’ with various behavioural or hormonal treatments. Isolation of chicks or treatment with the hormones vasopressin or vasotocin [22] shifted the 15 min dip to between 20 and 30 min after training and corticosterone, ACTH, vasopressin, vasotocin and testosterone shifted the 55 min dip to as much as 95 min [22, 24, 25]. Pharmacological intervention studies confirmed that the biochemical/physiological bases of these stages were different in that each was inhibited or enhanced by specific types of drugs.

There is in fact another stage, even earlier at 1 min post training, where chicks pecked at the bead [18] suggestive of a sensory input stage prior to short-term memory. These 4 stages were also noted by Rosenzweig et al [44] training on 10% MeA, albeit each of the

different stages lasted for different durations. Again this points out that different variations of the one task protocol can produce different timings. Richard Andrew, with a task very similar to that at Monash, has also found evidence of these memory stages [1, 32].

Saliency of the training experience.

Novelty

It seems clear that the saliency of the learning trial and how relevant it is to the chick is important. What appear to be subtle differences to us, may in fact be very important differences to the chick. At Monash, we feel that the task cannot be regarded as ‘passive’ avoidance. The chicks generally actively avoid the bead by turning away or going to the back of the cage. At Monash, although not so much at La Trobe, the task has become one of discriminative avoidance – where the important feature is the choice of the chick on the retention test with trials 2.5 min apart. One difference between the passive and discriminative avoidance tasks is whether the bead is novel at training[4] and whether the chicks have had pretraining experience on the same bead used for the training trial. At Monash/La Trobe, the chicks are given the opportunity to peck at both red and blue beads during pre-training. In this way, we know that they will peck at the red bead as much as they do so at the blue bead. On training the red bead is now aversive, so the chick now knows that the red colour, which was attractive before, is now unpleasant (bitter). When given clean red and blue beads on test, it is not the shape of the bead but it the colour that is important. After weakly-reinforced training or drug intervention, when the birds forget they were trained on red, they revert to the pecking both the red and blue beads. So it is the colour and not the shape of the bead that is important.

At the OU, the chicks are pretrained with a small white bead, and the chicks have not seen the training trial bead, which is larger and shiny, before. Being ‘novel’, the bead may be more important to the chick and hence, it may lead to a ‘stronger’ memory of the aversive bead and it becomes now important not to peck at bead this size, shape and colour in the future. When this bead is ‘forgotten’ either because of weakly-reinforced training or because of drug intervention, the bird now regards it on test as another novel

bead, whereas in the Monash protocol, the reverts to pecking at the bead which was attractive before. The findings of Burne and Rose [3] support this interpretation – the novel bead being aversive is remembered for the longer time in the Monash protocol where the chicks have seen the bead before. This therefore results in the chicks remembering for longer when using weakly-reinforced training paradigms. Even with strongly-reinforced training, this difference in protocol may result in ‘differing points within the same biochemical cascade, ie the duration of the different memory stages will be different’.

Discrimination, pecking the blue bead. The timing of the presentation of the non aversive blue bead relative to the test on the training bead varies between Monash (2.5 min later) to 20 or 30 min later (La Trobe and OU). At La Trobe this timing is to avoid an carry-over effects of pecking at the red bead (see below (7)). However, the response to the blue bead straight after the red maybe important. If, as found at La Trobe, some of the chicks avoid the blue bead when tested close to training, it could reflect some aspect of confusion. On the otherhand, although we have not analysed the number of pecks given to the blue bead apart from the discrimination ratio, the number of pecks given to the blue bead may reflect how well the chicks remember. In an unpublished experiments, increasing the number of training trials with 100% MeA, by repeating the training at 15 min intervals during the first transient dip in retention (when the chicks peck), we found that after two training trials nearly all the chicks avoiding pecking at the red bead but after three training trials results in chicks which now avoid the blue bead as well. A different scenario of this is when the reinforcer on the training bead is changed to water at 15min resulting in the chicks pecking at the bead from now on [19].

Johnston and Burne (this volume) have found that memory for a yellow and black striped bead is consolidated into long-term storage when coated with 10% anthranilate, whereas training chicks on separate yellow and separate black beads was not. Yellow and black stripes as well as being very conspicuous are colours associated with danger, increasing the salience of the learning experience.

Apart from the differences seen between isolated and paired chicks, and the differences in the nature of the task itself, what other possibilities for variability are present in the two major versions of the training task used by Monash/La Trobe and the OU, differences which presumably produce alterations in the duration of memory ? In Table 1, we list some the details that may be the cause of the variability.

INSERT TABLE 1 ABOUT HERE

Important variables in strain and housing conditions (Table 1)

1. Strain of chickens

Commented on above.

2. Age post hatch

The exact age of the chicks maybe an important factor; the chicks used at Monash/La Trobe come from the poultry farm where they are hatched ‘in bulk’ and remain in the incubator until they are sexed into males and females and delivered to our laboratories. There is a possibility that some chicks hatch earlier than Monday night and would therefore be slightly older than one day old when sexed and delivered to us on Wednesday morning. The hatching of chicks at the OU is under better surveillance and chicks are $24 \text{ hr} \pm 6 \text{ hours}$ at the time of the experiment. Recently, both La Trobe and Monash received a delivery of chicks from the hatchery where the chicks had hatched 24 hours earlier than normal due to an oversight in the setting of the eggs. The chicks were noted by all experimenters to be more difficult to get to peck on pretraining, thus many more birds than usual were excluded because of either not pretraining or training. Normally, all chicks peck at pretraining. Typically at Monash, no birds are excluded for not pretraining and only 1 or 2 at the most are excluded for not training. This suggests that when hatching chicks in the laboratory, it is important to make sure the chicks are at least one day old.

INSERT TABLE 2 ABOUT HERE

Differences in pretraining, training, testing and drug injections (Table 2).

3. Pretraining

The purpose of the pretraining is to accustom the chicks to the experimenter presenting beads to them to peck at and to ensure the chicks are not frightened by the experimenter presenting the bead. The bead presentation is always from the same direction ie from the right hand front corner of their box, and is accompanied by gentle tapping on the front of the box with the forefinger. This takes place over a series of small holes (see figure) in the front of the box and this provides a visual flicker. Therefore the chicks associate the auditory and visual cue with presentation of beads. The beads are dipped in tap water and are likely to have a taste. The water is not neutral and does provide a taste to the chicks. At Monash the coloured beads are glass seed beads, originally found as ‘worry beads’ in Crete.

In the absence of a pretraining protocol, chicks do learn and avoid the bead on test, but there is greater variability in the level of retention [33]. Whether the beads in pretraining are wet or dry has been shown at the OU to be an important variable [4], but at Monash pilot experiments have shown no difference between wet and dry beads on pretraining (Gibbs, unpublished).

4. Training.

Anthranilate is found in some sunscreens and is a natural component of cocoa, coffee, grape, grapefruit, jasmine, lemon, lime, mandarin, strawberry and tangerine (<http://www.sigmaaldrich.com>) which explains why it smells of mandarin. Although the chicks must be able to smell the anthranilate, the smell maybe attractive at first as chicks approach and peck at the bead within the first one or two seconds of presentation. With

prolonged exposure, the smell could become aversive. Human experience shows that it is the bitterness on the tongue that is aversive. However, there are reports that chicks associate the smell of MeA with the aversive taste [3, 37], but if the smell was aversive of itself the chicks would not peck at the bead. This may reflect differences in the amount of anthranilate on the bead and whether the chick sprays it around on shaking its head. At Monash we take care to make sure there is no excess anthranilate on the bead and deliberately tap the bead on a paper towel prior to presenting it to the chick, it is important that the chick does not swallow the aversant.

5. Time interval between pretraining and training.

The Monash and La Trobe protocols leave a gap of at least 30 min between the end of pretraining and commencing training, at Sussex University a gap of 120 min is usually left. The reason for doing this is to ensure that there is no interference with pretraining and training experiences [2, 8, 9] .

At the OU there are 3 pretraining trials on a small white bead before the training trial, the trials including the training trial, are spaced 5 min apart. As mentioned above, it may be important in the OU protocol that the training bead is novel so the birds have not seen to it before, whereas at Monash and La Trobe with discrimination training, both pretraining and training are done with the same bead and therefore it is not a novel bead. If the pretraining and training trials are too close – it is possible that the entire procedure could be regarded as one trial and if all trials are within short-term memory when the next is presented, the effect of each could influence the perception of the next presentation.

6. Concentration of anthranilate

As mentioned in the introduction, the concentration of anthranilate on the bead is important. At both La Trobe and Monash, 20% anthranilate is used in weakly-reinforced training, although it should be noted that Nikki Rickard and others in the Psychology Department at Monash have used 10% anthranilate for weakly-reinforced training since

1995. The reason for choosing 20% originally [12] was that this concentration of anthranilate was close to the threshold concentration needed to produce consolidation into long-term memory and therefore in this paradigm drug manipulations to consolidate memory required only minimal doses of drug to produce a detectable inhibitory or facilitatory effect. At Monash and La Trobe it has been found that two trials with 20% anthranilate (one at zero time and one at 15 min) are sufficient to reach the threshold and lead to memory consolidation [12]. With 10% anthranilate SFC and MG have found that it takes at least 3 trial repetitions before memory consolidation occurs [12] Gibbs unpublished). In respect of possible effects of pecking alcohol on the bead, there is no inhibition of pecking even with 100% ethanol on the bead and even with presentations spaced 10 min apart.

As mentioned above, the amount of anthranilate on the bead is an additional variable in strongly-reinforced training. It is possible to just leave a enough to deliver the taste to the tongue or to 'load' up the bead so that chicks get a mouthful (beakful) and some chicks can even swallow it.

A further confounder may be whether the anthranilate is dissolved in 100% ethanol or 40% ethanol diluted with water. At Monash and La Trobe the MeA the dilutions are made in pure ethanol which by itself is not aversive. Anthranilate is virtually insoluble in water and forms an emulsion with tiny droplets of MeA easily seen by eye. This emulsion separates out on standing. So if there is any water in the anthranilate mixture it is very important to shake the bottle well before dipping the bead, between each pair of chicks. This problem becomes more important if the water to alcohol ratio is increased further by going to 10% rather than 20% MeA and may be of importance in the dilutions in water used by Cherkin. Related to this, is the finding that with 33% MeA the chicks avoided the bead 2 or 3 hr after training [13] they did not avoid the bead at 24 hrs (Gibbs, unpublished 73% vs 39% avoidance). However, the discrimination ratio indicated that the chicks did remember (0.90 ± 0.06 at 2 hr; 0.81 ± 0.05 at 24 hr). This experiment suggests that it is the strength of the MeA as well as the retention measure that is contributing to the discrepancy between laboratories. The size of the MeA droplets in the emulsion is different, depending on the length of time and intensity of shaking, and on the delay

between shaking and dipping the bead – so care must be taken to keep procedures consistent.

7. Timing of the discrimination test.

There is debate between the different groups about when the neutral bead should be presented after test. At Monash neutral (blue) bead is presented 2.5 min after the red bead. Ideally, chicks should make the discrimination with both beads presented together, but for technical reasons this is difficult. Both at the OU and in the La Trobe avoidance task the neutral bead, when used, is presented 20 to 30 min post test. At La Trobe it is said that this avoids any ‘carry-over’ effects – this could occur, it is argued when the response to the test bead influences the response to the neutral bead [10]. This notion came about from experiments at La Trobe where it was reported that at test 10 min after training, although the chicks avoided the red bead, there was also some tendency to also avoid the blue bead. Earlier experiments, eg [21] had not shown increased avoidance of the blue bead close to the training trial. This matter is therefore not resolved, although as mentioned above the amount of MeA on the bead at training may have an effect. In early experiment both Art Cherkin in the US and June deVaus at La Trobe found that when MeA was put in the bill on a cotton bud without the chicks seeing it, pecking was inhibited for up to 5 min after training.

8. Injection procedures

The coordinates of injection for IMM used at Monash and the OU are very similar. The OU use a head holder [16] to precisely position the injections, whereas freehand injections are used by Monash. Freehand injections are obviously much faster and the injection can be done close to the same time that it takes to train a pair of chicks. Therefore at Monash the timing is very accurate and is done without the additional stress of having to place the head of the chick in a head holder. However, it must be admitted that there is considerable training required before accurate placement of injections can be achieved freehand and with a new or untrained investigator this variability has to be

weighed against the time and stress disadvantages of using a head holder. Sandi and Rose [45] point out the use of the modified head holder of Davis et al [16] is itself stressful and note that ‘chicks could not be injected earlier than 5 min after training without the injection procedure itself resulting in amnesia’.

INSERT TABLE 3 ABOUT HERE

Comments on differences in retention scores (Table 3).

9. Retention Scores.

The pre-training and training protocol at Monash results in chicks *tending* to avoid rather than *completely* avoiding the training bead at test. The advantage of this is that we do not hit ceiling of avoidances on red and routinely have memory scores between 1 and 0.5. In early experiments up to 1989 [23] we used % avoidance as the memory retention score even though % avoidance of blue beads were also calculated, but we (Gibbs) find that there is more information when a score based on number of the pecks on red and blue bead is considered. We now use the discrimination ratio and a further advantage of this ratio is that it is amenable to parametric analysis (see below).

La Trobe University has recently introduced a second way of measuring memory retention an ‘avoidance score’ as opposed to a ‘discriminated avoidance score’, this is the ratio of the number of pecks on pretraining to a red bead (no blue bead is presented) to the number of pecks on the red bead at test.

As discussed above, in discrimination training at La Trobe at the OU, when a coloured bead discrimination is used in an experiment, the neutral blue bead is presented 20 min after the red bead. This is different to the two trials being spaced 2.5 min apart. Simon Crowe showed evidence of a ‘carry over effect’ to the performance on the blue bead 5 and 10 min after the red aversive bead (with no intervening clean red bead). Although this has not been reported for other studies where memory for red and blue beads was tested 5 or 10 min after training (or even 1 to 5 min after training [18]), nonetheless, it

may have significance in the reported study [10] indicating that the chicks were not discriminating and responding to shape rather than colour at these short intervals when the blue bead was tested first.

10. Statistical analysis.

When only one type of bead is used at test, the choice is between whether a chick pecks or not and memory retention is the percentage of chicks avoiding and not pecking at the bead— the assumption is made that they are avoiding because they remember that the bead tasted nasty when it was pecked before. As mentioned above, the percentage avoidance score is not amenable to parametric analysis. Non-parametric statistics, while being more conservative, may also miss important information. Both Discrimination Ratios and the Avoidance scores are amenable to standard ANOVA testing.

Conclusion

There are real differences in the results obtained with the different pretraining/training protocols at the different universities, but they are not major and depend on what is being looked for. Since all investigators alter protocols because they see experimental advantages in changing the original protocol it is not clear that any one groups will change its procedures! All that can really be hoped for is that each group properly documents its methods and researchers both in and out of the field are aware of the differences in protocols in the literature.

One of the main differences between the ‘passive’ and the ‘discriminative’ avoidance tasks is whether the birds avoid the bead characteristics used in training (passive avoidance) or peck less at that bead than a neutral one, discriminating between the colours (discriminative avoidance). It could be argued that the task is not ‘passive’ as the birds do not just sit and ignore the bead.

Another important difference, is whether the bead used on training is novel - whether the chicks have experienced the particular colour, both the shape and size of the bead before,

and in discriminative avoidance task when they forget they revert to pecking at the bead which was not aversive before. Additionally, the chicks can discriminate between shape or colour, features which may impact on the salience of the learning task. Small beads are more attractive than larger beads. In the OU protocol the bead used for training is novel, both its shape and colour (silver) are novel, the chicks have not seen it before, whereas in the Monash protocol the chicks have seen both beads and 'know' prior to training that the red and blue beads are OK to peck. After training, the decision to peck or not is based on colour discrimination as the beads are identical in shape. In the OU, and also the La Trobe protocol, whether or not the chicks will peck at a different bead is tested some 30 min later. At the OU, this discriminant bead is the one used in pretraining, whereas in the La Trobe protocol the discriminant bead is a new ie novel colour.

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A comparison of protocols for passive and discriminative avoidance learning tasks in the domestic chick.

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TABLE 1: CHICK SOURCES AND HOUSING CONDITIONS

	MONASH UNIVERSITY (<i>LA TROBE UNIVERSITY</i>)	OPEN UNIVERSITY
Source of chicks	<p>(i) Local Poultry Farm (Wagners Poultry Farm, Coldstream, Victoria), where they are transferred from incubator to hatchery around day 18 and hatched under red light. Eggs are exposed to light exposure on transfer from incubator to hatchery.</p> <p>(ii) Hatched in laboratory in either Brinsea or Multi-Quip self-turning incubators for developmental experiments, the chicks are kept in the incubators until completely dry (12 - 24 hours) and then in dim light under brooder lamps for 24 hours- then treated in the same way as those that come from the Poultry Farm.</p>	<p>Fertile eggs from Ross Chunky broiler-strain chickens (Maurice Millard Chicks, Trowbridge, U. K.) are incubated for the first 17days in a communal brooder maintained at 37.5 – 37.9°C and on an 8:16h light/dark cycle.</p> <p>On day 18 of incubation the eggs are transferred to a hatching trays (~20 eggs per tray) and moved into a hatching incubator held at 37–38°C and maintained on a 12:12 light/dark cycle and allowed to hatch.</p>
Chick strain	Egg laying strains, a hybrid of White Leghorn, New Hampshire, Black Australorp and Rhode Island Red. Chicks weigh around 35 gm at hatch. No differences have been seen between these strains	Ross Chunky broiler-strain chickens (Maurice Millard Chicks, Trowbridge, U. K.)
Delivery	By private transport vehicle, and in standard chick transport cartons – the chicks are not distressed by the transport and are usually sleeping on arrival.	The chicks are removed when dry from the group hatching tray in the dimly lit hatching incubator and transferred to a large (60cm x 60cm x 25cm) aluminium holding cage, maintained at ~28°C where food and water are available ad lib
Sex	<p>Generally males, but unsexed on occasions and unsexed when laboratory incubated. No difference in behaviour or memory characteristics have been seen between males and females, although one would be wary if investigating the effects of sex hormones.</p> <p><i>La Trobe use males only as the University Ethics Committee will not allow the use of unsexed chicks</i></p>	Both males and females, where required chicks were sexed by trained operatives inspection of the gonads.
Age of chicks	Eggs are set at the Poultry Farm on Monday evenings, chicks hatch out Monday evening to Tuesday morning 21	24 +/- 6 h after hatching chicks are transferred, in pairs to pens

	days later. The chicks are sexed Wednesday morning starting at 8.00am and we collect them to start experimenting between 9 and 9.30. Chicks are one-day old and into their second day after hatch	
Housing conditions	Chicks are placed in pairs into wooden boxes (18 x 25 x 20cm) with holes on the front panel and maintained in pairs throughout the protocol. <i>La Trobe: boxes are 20x25x20cm</i>	Chicks placed in pairs in small aluminium pens (20 × 25 × 20 cm) floored with blue towelling paper
Box temperature	26 to 29°C. Heat source is 15W pilot globes (white) suspended above each cage, as well as general lab heating.	Illuminated by an overhead 10-W red pearlescent bulb. Heat is provided red light bulbs which maintain the pens at a steady 25-27°C.
	Chick crumbs <i>ad lib</i> scattered on the floor. Not very much of the food is consumed, the food is present on the floor as targets to peck at.	They are supplied <i>ad libitum</i> with chick starter crumbs scattered on the floor. Water was provided from a white open dish (~8cm diameter) situated at the rear of the pen, where it remained during equilibration period, training and testing
Procedure on Arrival	9.30am – placed in experimental boxes on arrival.	Chicks placed in pens and remain undisturbed in their pen for 1 h before training. This procedure is usually conducted between 9 and 11am in the morning.
Group sizes	Starting number of 16 chicks per group <i>La Trobe 20 chicks per group</i>	18-22 chick/group
Pairing	The variation in colour of the New Hampshire x Rhode Island Red chicks, pairs are selected on basis of darker and lighter. They are initially put into boxes in groups of 4 ¹ , within 20 min they divided into pairs, if necessary to discriminate between pairs chicks are marked with a (non-smelling) black marker pen. <i>One chick is marked on the head with a black permanent marker.</i>	One chick in each pair is marked on the back with a spot of blue non-toxic animal dye.

TABLE 2: PRE-TRAINING TRAINING AND TESTING PROCEEDURES

	MONASH UNIVERSITY (<i>LA TROBE UNIVERSITY</i>)	OPEN UNIVERSITY
Pretraining	<i>WATER ON SMALL CHROMED BEAD</i> Chicks are ‘pretrained’ in groups of 4 within 15-20 min of being placed in the cage- they receive a brief exposure to small, wet chromed bead which is left in cage for around 10 sec or until they peck. The intention of these procedures is to get the chicks used to their new boxes and bead presentations at the same time. This reduces the novelty of the bead presentation and the likelihood of the chicks being scared <i>La Trobe: this step is carried out in with the chicks in pairs</i>	<i>DRY SMALL WHITE BEAD</i> Following an equilibration period of one hour, chicks are pretrained by three 10 sec presentations of a small (2mm dia) white glass bead attached to a thin (1mm diameter) dull metal rod, at approximately 5 min intervals. Chicks that fail to peck the bead at least twice in three presentations (less than 5%) are not used subsequently, but remain in their pens for the duration of the experiment.
Second exposure to wet small chromed bead	Within 20 min they are sorted into pairs and pretrained briefly again with the small wet chrome bead <i>La Trobe: 20 minutes later, chicks receive a second pretraining trial with a water coated chrome bead</i>	
Pre-training on blue then red glass beads (plastic dissolves in methyl anthranilate)	Monash: 11.00 am. Chicks are presented with the blue bead for 10 sec followed 2.5 minutes later by the red bead for 10 sec. The latency to the first peck and the number of pecks for each chick are recorded on hand held electronic ‘logger’. Presentation of the blue bead first on pretraining results in equal pecking on blue and red beads. The discrimination ratio (ratio of pecks at blue and red beads) is only slightly biased toward avoiding the red bead on pretraining ($dr=0.55-0.6$). <i>La Trobe: Avoidance ratios: pre-training occurs on a red water coated bead only. Number of pecks is recorded. Discrimination ratios: pre-training occurs on water coated red and blue beads as described above. However the order of the bead presentation is randomised and an interval of approximately 20 minutes separates bead presentations. Number of pecks is recorded for each bead.</i>	
Training procedure	Training commences at 12.00. The red bead is dipped into	<i>Training is reported to start 5 min after the last</i>

	<p>20% or 100% anthranilate and then dabbed onto a paper towel to remove any excess anthranilate. The chicks register the taste on the tongue and do not swallow any of the anthranilate. Latency to first peck and number of pecks in 10 sec recorded. Bead left in the box for 10 sec.</p> <p>20% anthranilate is made from dilution of 100% in alcohol (100%). Alcohol itself on the bead does not result in avoidance of the bead at least over 4 trials.</p> <p><i>La Trobe: The red bead is wiped around the top of the MeA container to remove excess and prevent dripping into the box. Training can also occur on a water coated red bead to control for non-specific drug or protocol effects.</i></p>	<p><i>pretraining trial</i> and consists of a 30 sec. presentation of a 4mm dia. chrome bead been dipped in either the bitter-tasting methylanthranilate (MeA; strong training) or a 10% solution of MeA (weak training (WT): diluted in 40% ethyl alcohol). Chicks are always monitored for disgust response presentation.</p>
Drug injections	<p>Bilateral injections of 5µl are made into discrete sites in each forebrain using 250 µl Hamilton repeat dispensing syringes that give 50 injections per barrel. Placement of injections is determined using bony landmarks on the dorsal surface of the skull. The accuracy of siting of the injections is checked at the completion of the experiment by examination of the needle tracts in the cartilaginous skull.</p> <p>Subcutaneous injections of 100µl are placed into a ventral skin fold below the rib cage.</p> <p><i>La Trobe: Injection volume is 10µl bilaterally (i.e. 20µl total) and is targeted at the IMM as outlined above.</i></p>	<p>Bilateral intracerebral injections of peptide or saline (routinely 2µl) are made using a 10µl Hamilton syringe fitted with a plastic sleeve, in most experiments organised so as to allow a penetration of 3.5mm designed to deliver the injection into the intermediate medial mesopallium (IMMP, previously called IMHV [36], a region known to be crucial for memory formation [41]. The headholder requires insertion of the chick's beak into a specially created groove, enabling needle access through carefully positioned holes in the top of the holder for intracranial injections. Because of the chick's unossified skull intracerebral injections do not require anaesthesia. These injections are rapid (<20 sec per bird) and cause no observable distress to the chicks. All procedures were carried out under Home Office Licence to SPRR and are classified as Mild.</p>
Co-ordinates	<p>A plastic sleeve on the 27 gauge needle controls the depth to 3.5mm (from tip, the orifice is around 1mm).</p> <p>IMM injections are ~3 mm left and right of midline and 4-5mm from ridge on skull dividing cerebellum from forebrain. In a typical experiment the accuracy of this freehand</p>	<p>Correct placement of injections into the IMMP is ensured using a custom-built headholder [16] and a plastic sleeve on the Hamilton syringe needle to control the depth of injection to 3.5 mm . IMM injections are 2.5 mm left and right of midline using.</p>

	placement was 3.30 ± 0.14 and 2.73 ± 0.14 mm to the left and right of the midline and 4.6 ± 0.18 and 5.00 ± 0.17 mm from tegmentum.	plates 7.6-7.8 from the atlas of Kuenzel and Mason, [30]. Injection sites are routinely visually monitored post-mortem.
Conditions between training and testing	Chicks remain in cages until testing, conditions are not changed from above	Following training chicks remain, in pairs, in these 'pair' cages for a variable time (usually 24 hours) prior to testing. The room is kept on 12/12 hr dark/light cycle
Testing	<p>For 120 min training-test interval tests made commencing at 2.00 pm. Chicks are tested on red then blue with 2.5 min interval. Chicks are allowed 10 sec to peck</p> <p><i>Avoidance ratios: Chicks are tested on a dry red bead only.</i></p> <p><i>Discrimination ratios: Chicks are tested on a dry red bead followed by a dry blue bead approximately 20 minutes later to avoid generalised avoidance to the previously non-aversive bead.</i></p>	<p>At the appropriate time following training chicks are tested by offering them a dry 4mm dia. chrome bead, followed at least 10 min later (up to 30 mins later) by a small (2mm dia.) white bead, each bead presented for up to <u>20-30</u> sec. Each chick is trained and tested only once, normally by an experimenter blind as to prior treatment of the birds.</p> <p>The white bead is the same as that used in pretraining and the chicks are given 20 or 30 sec to peck</p>

TABLE 3. DATA COLLECTION AND ANALYSIS.

	MONASH UNIVERSITY (<i>LA TROBE UNIVERSITY</i>)	OPEN UNIVERSITY
Data collection	Data loggers record the latency to the first peck for each chick on introduction of the bead into the box, and the number of pecks given by each chick in the 10 seconds. Chicks have been recorded as giving up to 25 pecks in the 10 seconds. Data downloaded onto computer and analysed at the completion of the experiment	By hand by experimenter blind to the treatment of the chicks. Only whether the chicks peck or not is recorded except where discrimination data is recorded
Elimination of chicks	Exclusion of chicks from analysis – 1. Chicks not pecking on training – not regarded as training unless they peck the red bead even if they head shake 2. Chicks not pecking on the blue bead are removed because of the possibility of generalized avoidance or non-specified performance effects. There could be something wrong if the number of chicks avoiding the neutral bead is greater than two or three chicks in a group of 16 chicks. <i>La Trobe Avoidance Ratio: With the avoidance ratio measure chicks are only excluded if they do not peck on the training trial</i>	Only chicks that peck at the bitter bead on training and the white bead at test are included in the final results.
Retention score	Discrimination ratio – the number of pecks on the blue bead divided by the number on the red bead plus the blue bead. <u>Pecks on blue bead</u> Pecks on red and blue beads When chicks avoid the red bead the discrimination ratio is 1. When chicks peck the red and blue beads equally, the discrimination ratio approaches 0.5. Therefore NO memory is indicated by a discrimination ratio of 0.5. This is then used as the zero point on the Y-axis of the graph. <i>Avoidance ratios: Chicks are tested on a dry red</i>	Chicks are considered to remember the task if they avoid the chrome bead at test, but peck at the white bead, and to have forgotten it if they peck at both beads. Amongst the chicks that met this criterion (>80%), recall is calculated as a percent avoidance score (i.e number of chicks in each group that avoid the chrome bead but peck the white on test X100/ Total number of trained chicks) or as discrimination ratio between white and chrome bead

	<i>bead only.</i> <i>Discrimination ratios: Chicks are tested on a dry red bead followed approximately 20 minutes later by a dry blue bead to remove the possibility of generalised avoidance to the previously non-aversive bead.</i>	
Statistical analysis	<p>One or two-way analysis of variance depending on the experimental design, using discrimination ratios and comparing experimental with control groups using SPSS. Significance level of $P < 0.05$.</p> <p><i>La Trobe: as for Monash for both DR and AR but using post hoc univariate Dunnett's test for comparing each drug treatment group with the vehicle control.</i></p>	<p>The differences between groups are tested for statistical significance by G-test for goodness of fit and the Williams correction [48] to the observed G. Chi-square criteria are used to determine whether the behavioural response (peck vs. avoid) of chicks in treated groups differed from that in the control group(s). Significance levels are set at 5% or avoidance data analysed as per LaTrobe by one-way analysis of variance</p>